EXHIBIT D

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Polyethylene glycol–conjugated pharmaceutical proteins

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Polyethylene glycol (PEG)–conjugated proteins belong to a new class of biomolecules that are neither proteins nor polymers, but which are hybrids of the two. PEG conjugation of biopharmaceuticals is now common practice in efforts to achieve sustained clinical response. However, very little is known about the strategies and criteria used to produce a well-characterized pegylated biomolecule. In this review, the issues of pegylation reagent selection, reaction conditions, process considerations and purification will be addressed, as well as biochemical and biological characterizations. The isolation of positional isomers and the determination of pegylation sites will also be included.

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Recombinant proteins are noted for their short circulating half-life in blood. This shortcoming is accompanied by reduced bioavailability and consequently, reduced clinical potency. In 1977, Abuchowski and colleagues demonstrated that, as therapeutic agents, polyethylene glycol (PEG)–conjugated proteins are more effective than their corresponding unmodified parent molecules[1]. Since then, several pharmaceutical proteins have been pegylated and have been shown to have properties of use in clinical applications[2–5]. These improved clinical properties include better physical and thermal stability[6–8], protection against susceptibility to enzymatic degradation[9–11], increased solubility[12–14], longer in vivo circulating half-life[15–17], decreased clearance and enhanced potency. Three other properties of pegylated proteins are reduced immunogenicity[1,2,18] and antigenicity[1,2,19,20], as well as reduced toxicity[19,21]. A further effect of pegylation is reduced in vitro activity accompanied by enhanced in vivo activity. This effect has been observed with granulocyte–macrophage colony stimulating factor (GM-CSF) (Ref. 20), interleukin-2 (IL-2) (Ref. 14), tumor necrosis factor-α (TNF-α) (Ref. 31), IL-6 (Ref. 32), CD4–IgG (Ref. 33) and interferon-γ (IFN-γ) (Ref. 34), among others.

Rationale

In addition to the aforementioned beneficial properties of pegylated proteins, pegylation can impart the following advantages:

- altered biodistribution;
- altered biological properties[35–37] (see Table 1);
- enhanced membrane penetration[31], sustained clinical response with minimal dosing leading to improved quality of life and reduced treatment cost.

Conceptual considerations

In general, PEG-conjugated biomolecules exhibit physicochemical properties that are different from those of the parent molecules. These properties include conformational changes, sterical interference, changes in electrostatic-binding properties, hydrophobicity, local lysine pKas (a measurement of the degree of completeness of a reversible reaction) and pI (the pH at which a protein's charge is neutral). Binding affinities to the receptors are often affected by these physicochemical changes, resulting in reduced activities in cell-based assays, in which incubation times are usually short. There is a direct relationship between the attached PEG mass and the in vitro biological activity[19]. In contrast, an inverse relationship exists between the PEG mass and the in vitro activity[32]. These relationships are illustrated in Fig. 1. Pegylation results in the retardation of renal clearance, which in turn prolongs the circulating half-life of the molecule and increases the area under the curve. The increased biological activity is attributed to these phenomena. In cell-based in vitro assays, because incubation is performed for a fixed time period, the enhanced half-life of the pegylated biomolecule has no role

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Table 1. Altered biological properties of pegylated biopharmaceuticals

PEG–proteins	Altered bioactivity	Reference
GM-CSF	Neutrophil priming[a]	Knusli *et al.*[35]
	Colony stimulating[a]	
IFN α-2a	Antiviral[b]	Pallerani *et al.* (pers. commun.)
	Antiproliferative[b]	
IL-15	CTLL proliferation[b]	Pettit *et al.*
	Antagonistic[b]	
Cholesterol esterase	HDL cholesterol[b]	Suguichi *et al.*[37]
	LDL cholesterol[b]	
Cholesterol	HDL cholesterol[b]	Suguichi *et al.*[38]
	LDL cholesterol[b]	

[a]Increased bioactivity.

[b]Decreased bioactivity.

Abbreviations: PEG, polyethylene glycol; GM-CSF, granulocyte–macrophage colony stimulating factor; IFN, interferon; IL, interleukin; CTLL, cytotoxic lymphocytic leukemia (a murine cell line); HDL, high density lipoprotein; LDL, low density lipoprotein.

to play. The relatively short incubation time, combined with the low receptor binding affinity of pegylated proteins, cause a lowering of in vitro bioactivity.

PEG attachment: unisite vs multiple sites

It is estimated that an optimal PEG mass of 40–60 kDa is required to retard renal and cellular clearance[5,7,18]. The attachment of PEG to protein can be achieved in three different ways: a single large PEG at a single site, a branched PEG (two- or more medium PEG chains joined together via a linker) at a single site, or several small chains at multiple sites. In theory, unisite pegylated proteins should have higher activity because of a reduced chance of PEG attachment occurring at receptor-binding domains. Multiple sites of pegylation or the attachment of large PEGs may result in partial- or complete loss of bioactivity.

Overlapping bioactivities

Potentially, there is an advantage in having a PEG–protein conjugate mixture comprised of unmodified, mono-, di-, tri-pegylated and other higher forms of protein PEG–conjugates. In theory, the higher the degree of pegylation, the slower the rate of absorption (prolonging the duration of circulation) and receptor saturation. The overlapping activity expected from such a mixture because of various absorption rates is illustrated in Fig. 2.

Polyethylene glycols (PEG)

Polyethylene glycols are amphiphilic polymers comprised of repeating ethylene oxide subunits, whose number is represented by the whole integer n (Fig. 3). Each ethylene oxide residue has a molecular weight (MW) of 44 Da, and n × 44 Da represents the number average MW of the PEG chain. Polyethylene glycols are inert, nontoxic and contain two terminal hydroxyl groups that can be chemically activated. However, it is common practice to convert one of the two hydroxyl groups to methoxy or other alkoxy groups, in order to make the PEG unifunctional. In addition to linear PEG chains, there are also branched PEGs in which two or more PEG chains are joined together with linkers such as lysine[41] and triazine[1], among others. Figure 3 illustrates some representative PEG molecules.

Pegylation chemistries

A variety of pegylation chemistries and reagents are now readily available[18]. They differ in their relative chemical reactivity

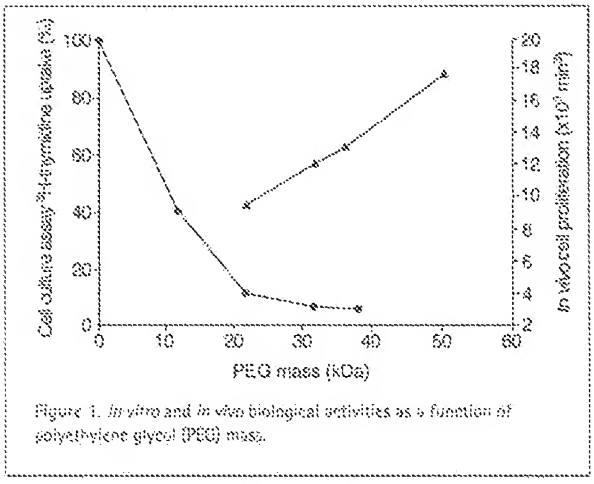

Figure 1. *In vitro* and *in vivo* biological activities as a function of polyethylene glycol (PEG) mass.

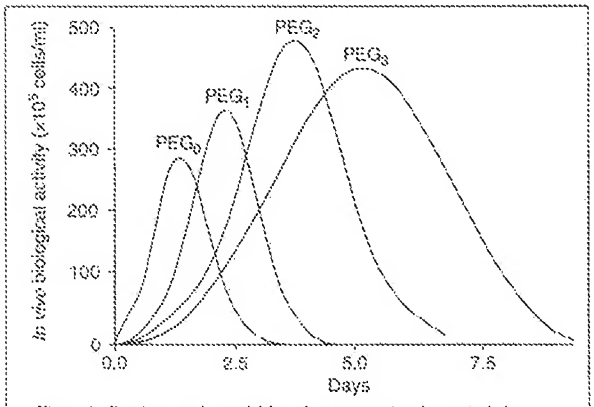

Figure 2. Overlapping bioactivities of components of a polyethylene glycol (PEG) conjugate mixture.

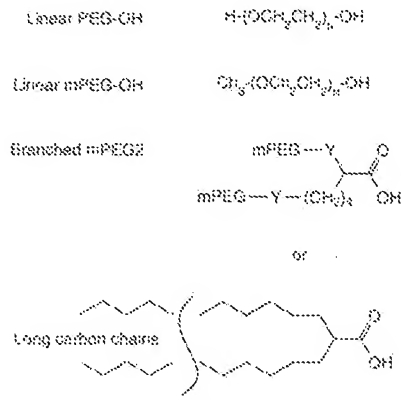

Figure 3. Chemical structures of some representative polyethylene glycol (PEG) molecules

Figure 4. A typical solution-phase pegylation process involving reagents such as N-hydroxyl succinimide ester derivatives of polyethylene glycol (PEG). The pegylation reaction is performed at a protein: reagent ratio of 1:3–5 for 8–16 h at 4–8°C, pH 7–8. After acidification and dilution, the PEG conjugate is purified on a cation exchange column. See text for details

and specificity. The most commonly used pegylation reaction involves an electrophilically activated PEG and the ε-amino group of lysine or the protein's N-terminal amino group. Examples include:

- N-Hydroxysuccinimide-activated esters[29,31–36] (amide bond);
- PEG-Epoxide[38] (alkyl bond);
- PEG-Carbonyl imidazole[37] (urethane bond);
- PEG-Nitrophenyl carbonates[39] (urethane bond);
- PEG-Tresylate[40] (alkyl bond);
- PEG-Aldehyde[38] (N-terminus, Schiff's base).

In addition to the attachment of PEG via primary amino groups which often produces heterogenous PEG conjugates, site-directed pegylation of proteins can be achieved via other functional groups on the protein surface. These include, free cysteines, oligosaccharides and alcoholic groups. PEG reagents used for site-specific pegylations are:

- PEG-Vinyl sulphone[51] (via free cysteine);
- PEG-Iodoacetamide[52] (via free cysteine);
- PEG-Maleimide[53] (via free cysteine);
- PEG-orthopyridyl disulfide[54] (via free cysteine);
- PEG-Hydrazide[55] (via oligosaccharide);
- PEG-Isocyanate[56] (via alcohol or amino group).

Pegylation reaction parameters

Factors that affect pegylation reactions are:

- reaction pH;
- protein to PEG-reagent molar ratio;
- protein concentration;
- time of reaction;
- reaction temperature.

By controlling one or more of these factors, the reaction can be directed towards producing predominantly mono-, di-, tri-, etc... PEG conjugates.

Pegylation process considerations

The pegylation of proteins can be performed in solution-phase or in a continuous solid-phase mode.

Solution phase

In a typical solution phase pegylation reaction involving PEG reagents such as N-hydroxysuccinimide esters, the final protein-to-reagent molar ratio used is usually 1:3–5. The reaction pH is maintained at around 7–8, the temperature is kept at 4–8°C and the reaction is terminated after 8–16 h by adjusting the pH to 4.5 with glacial acetic acid. The reaction mixture is then diluted with water and adsorbed onto a cation exchange column. Excess reagent and reaction byproducts are washed away. Pegylated protein is then separated from the unmodified protein by using increasing concentrations of salt in the buffer. A typical purification scheme is illustrated in Fig. 4.

Solid-phase pegylation

In solid-phase pegylation[57], the protein to be pegylated is adsorbed onto an anion exchange column and the PEG reagent is circulated through the column for a predetermined period of time. Excess reagent and other reaction byproducts are removed from the column by washing with buffer. The salt concentration

of the elution buffer is chosen so that only the pegylated protein elutes, while the unmodified protein remains on the column. From the eluate, the amount of protein pegylated is determined and the column is replenished with an equivalent amount of unmodified protein, so that the original amount of protein is maintained at the start of the next cycle of operation. PEG reagent is circulated again and the modified protein is separated from the unmodified as in the first cycle of operation. In the solid-phase pegylation process, reaction, separation and purification are performed on the same column and can be repeated as many times as required.

Biochemical and biological characterization

Protein determination

Protein concentration is determined by standard protein assay methods such as, UV absorbance, Lowry, bicinchoninic acid (BCA) (used in protein assays for color development), Bradford, etc., or by the more reliable and accurate amino acid composition-analysis method.

SDS-PAGE analysis

SDS-PAGE is performed according to the methods of Laemmli[55], and the gels can be stained specifically for protein or PEG. Staining for PEG is done according to a modified procedure of Kurfurst[56]. SDS-PAGE gels are rinsed with distilled water and placed in a 5% barium chloride solution for ten minutes. After rinsing the gel with distilled water to remove barium chloride, the gel is placed in 0.1 M Tirisol™ (EM Science, Gibbstown, NJ, USA) for another ten minutes. After washing away the yellow Tirisol™ (iodine solution), the gel is stored in distilled water in a heat-sealed Kapak/Scotchpak bag in the dark.

Molecular weight determination and identification

Mass spectrometry. Though not quantitative, mass spectrometry is an ideal tool in determining the true MW of various PEG conjugate species. Matrix-assisted laser desorption time of flight mass spectrometry (MALDI TOF MS) is now an established method for not only determining the true MW of PEG conjugate species, but also in identifying the individual species contained in a particular preparation[60,61].

SDS-PAGE analysis. Because of the anomalous (retarded) electrophoretic mobility of PEG moieties, SDS-PAGE analysis gives higher than expected MW, when compared with MW marker proteins. In order to circumvent this problem one can use PEG MW standards, and stain specifically for PEG.

Determination of pegylation sites and positional isomers[60,61]. Pegylation sites and the corresponding positional isomers are determined using a combination of techniques. These techniques include, ion exchange HPLC, size-exclusion chromatography, reversed-phase HPLC, peptide mapping, Edman sequencing, amino acid analysis and MALDI TOF MS. The potential number of positional isomers in a pegylated molecule can be calculated using the following factorial equation[59]:

For N pegylation sites, taken k at a time,

$$\frac{N}{(N - K)! \times K!} = \text{possible number of combinations}$$

Bioactivities

As illustrated in Fig. 1, there is an enhancement in in vivo biological activity relative to the PEG mass attached, whereas a decrease in in vitro activity is observed with an increase in PEG-mass attachment[39]. It should be pointed out that in almost all cases, the in vitro activity is not predictive of the in vivo activity. For reasons explained in the section on conceptual considerations, in vivo activity is many fold higher than that observed in an in vitro assay.

Pharmacokinetics

When injected into animals, pegylated biomolecules exhibit superior pharmacokinetic properties than those exhibited by the unmodified parent molecule. The biological activity is long-lasting because the serum circulating half-life and the plasma residence time of a PEG conjugate are often many fold higher than those of the unmodified molecule[17–22].

Immunogenicity

In immunogenicity a much reduced antibody response is observed with pegylated proteins when compared with those observed with an unmodified- or aggregated form of the unmodified protein[1,23–28].

Toxicity

In terms of toxicity, overall pegylated proteins are less toxic than the unmodified parent molecule[19,29]. However, toxicities have been observed with topically applied low MW PEGs[62]. These adverse effects are not observed however, when low dosages of PEG are administered orally or parenterally[63]. Because only chronic administration of large quantities of PEG elicits such toxicities, it is not expected to be the case with pegylated proteins such as cytokines, enzymes and hormones.

Tissue uptake of polyethylene glycol

Small PEG's tend to translocate freely from circulation into extravascular tissues and diffuse back into circulation[19]. However, a much slower diffusion process is observed for large PEG molecules. The larger the size of the PEG, the lower the renal clearance. In contrast, liver clearance increased with

higher MW PEGs. When the MW of the PEG is >50 kDa, the uptake by Kupfer cells is enhanced.

Regulatory guidelines of pegylated biopharmaceuticals

When developing pegylated biomolecules as therapeutic agents, the following points should be considered:

- starting materials (protein and PEG reagent) should be well-characterized;
- pegylated proteins are a new class of molecules;
- pegylated proteins are heterogeneous: neither proteins nor polymers, but hybrids of the two;
- sites of pegylation and stoichiometry of PEG attachment should be determined;
- consistency of each preparation should be established;
- derivatization and manufacturing processes should be validated.

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